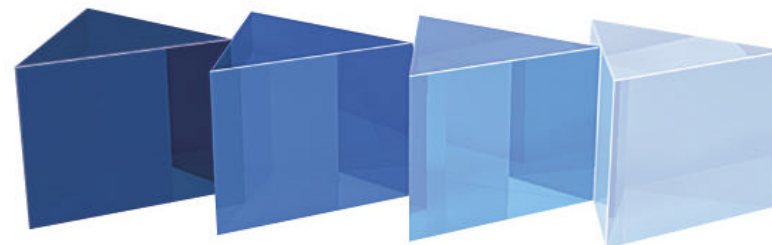


# Critical Factors For Successful Real-Time RT-PCR

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Dept. Modification/Amplification  
QIAGEN

Integrated Solutions



GENE*X*PRESSION

# Annealing

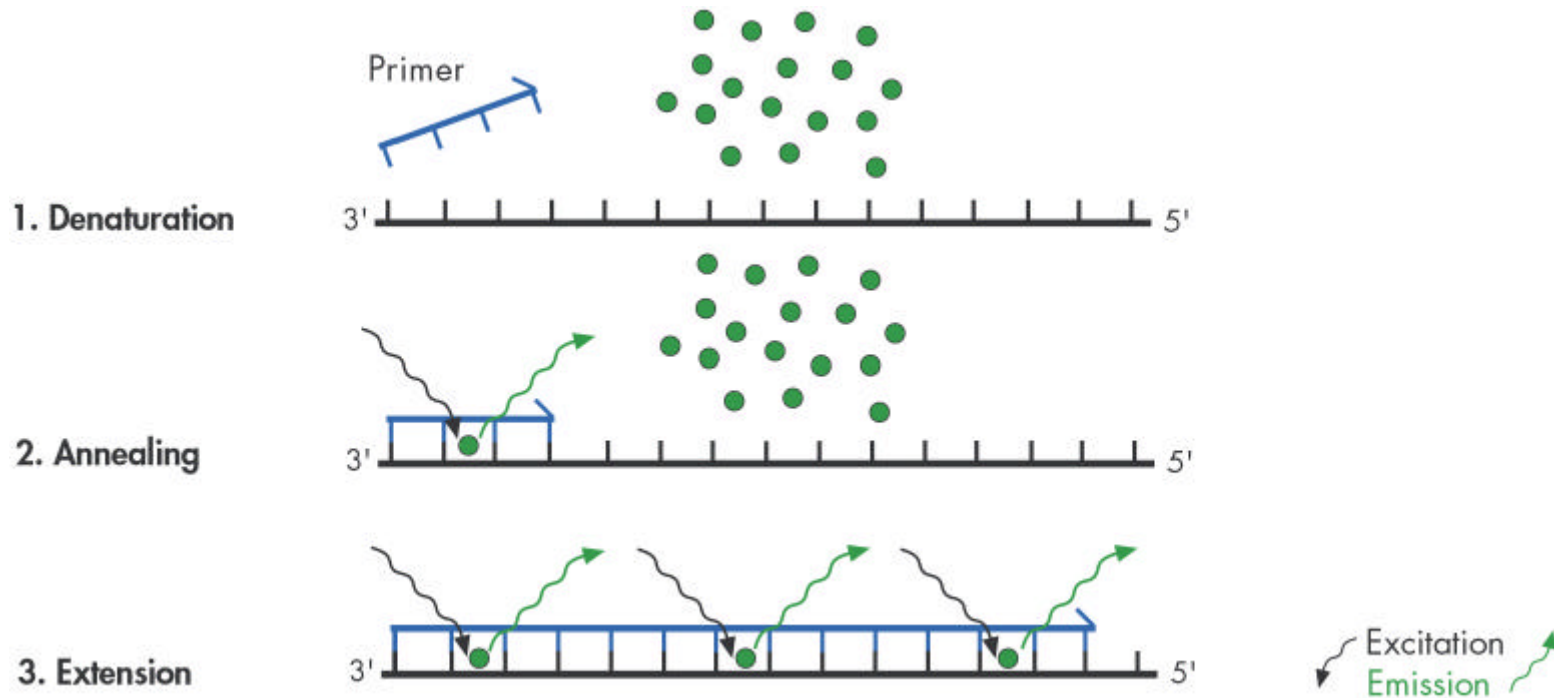
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Specific product  
High yield  
High sensitivity

Nonspecific product  
Low yield  
Low sensitivity

# SYBR Green Detection



→ Detection of specific & non-specific PCR products

# Specificity & SYBR Green Based Detection

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Non-specific PCR products result in:

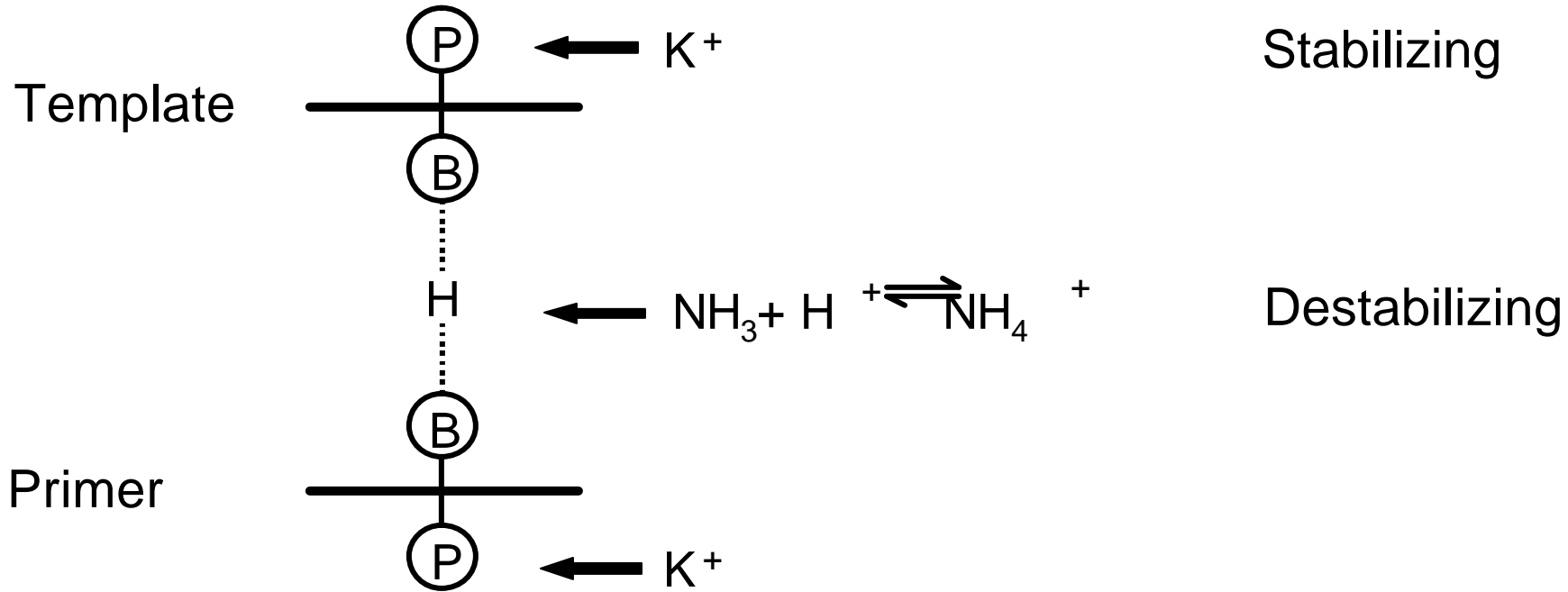
- Non-specific fluorescent signals (e.g. by primer-dimers)
- Reduced sensitivity
- Inaccurate quantification

# Factors Influencing PCR Specificity

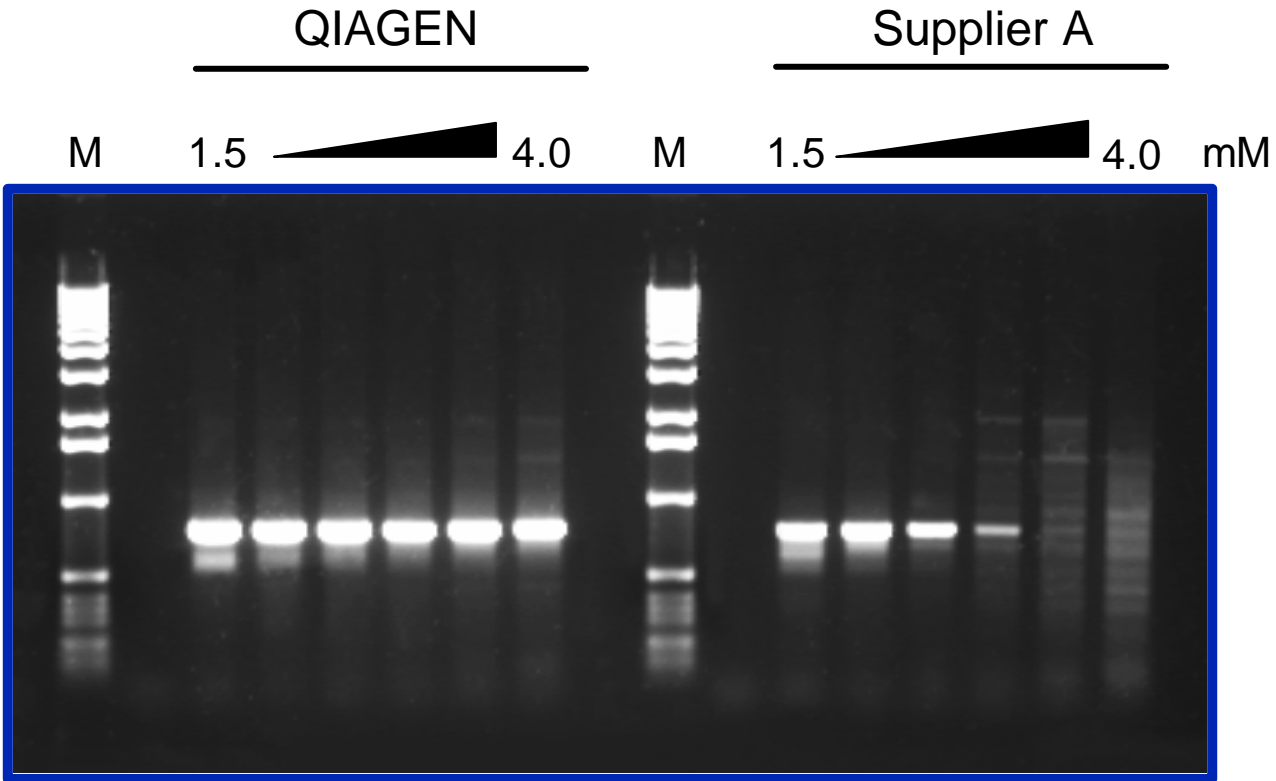
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- Amount of template
- Primer design
- Cations
- Initial artifact generation by *Taq* DNA polymerase

# Effects of Cations

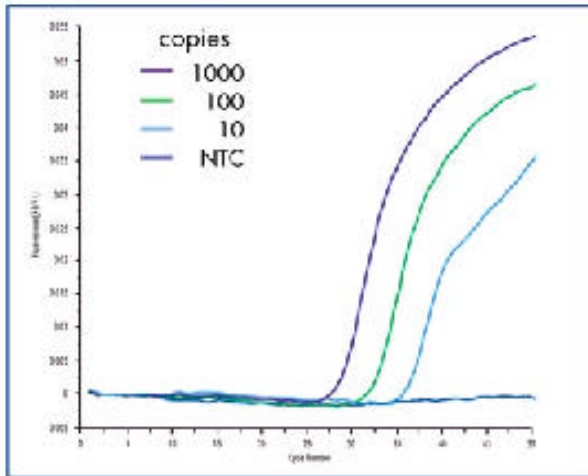


# Variable Mg<sup>2+</sup> Concentration

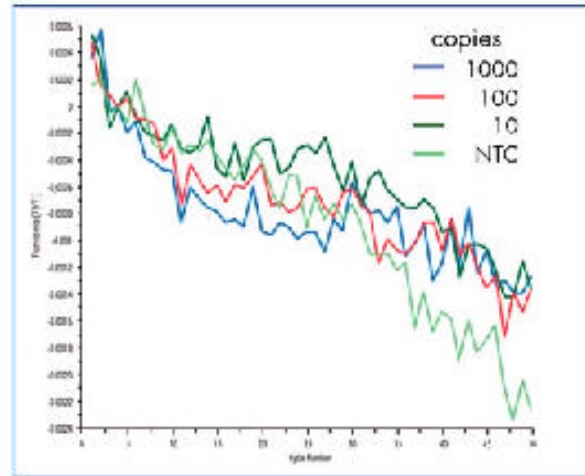


# Effect of Mg<sup>2+</sup> Concentration

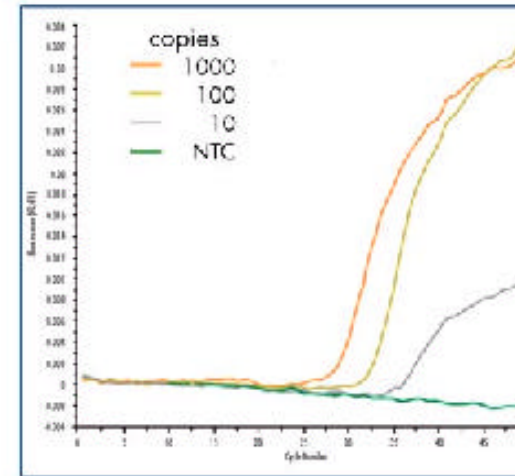
**A** QIAGEN,  
Mg<sup>2+</sup> concentration supplied



**B** Supplier R,  
Mg<sup>2+</sup> concentration supplied



**C** Supplier R,  
optimized Mg<sup>2+</sup> concentration





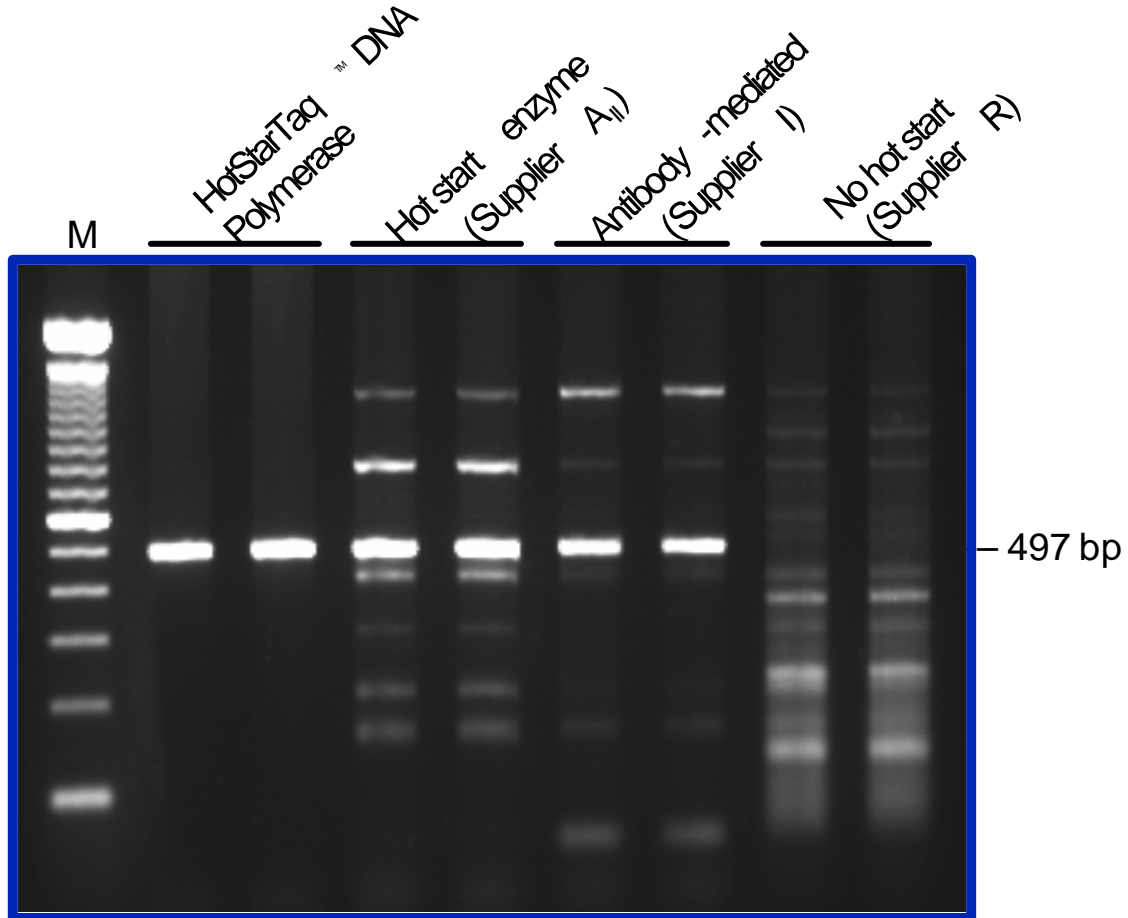
# PCR Specificity: Initial PCR Cycle

---

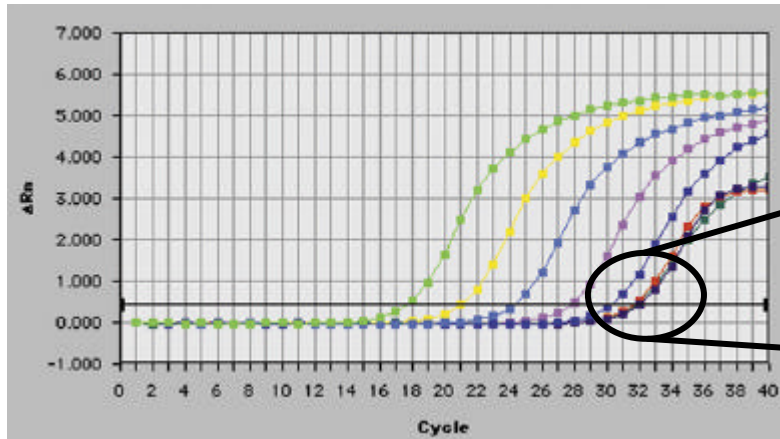
Nonspecific amplification starts during

- Reaction setup at room temperature
- Initial heating phase of thermal cycler

# Different Hot Start Methods



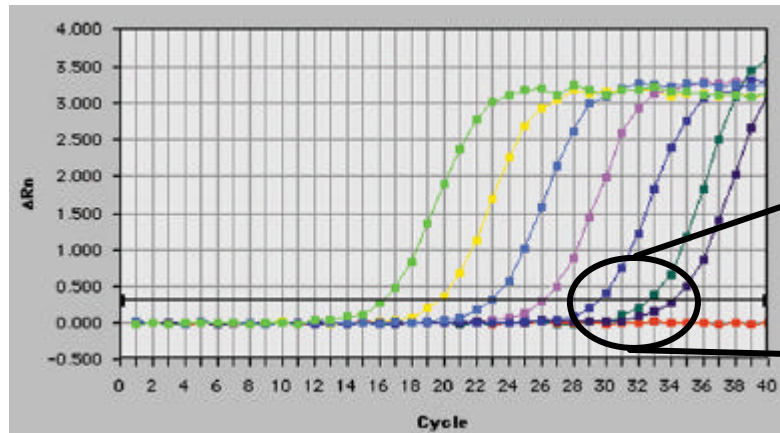
# Effect of Specificity on PCR Sensitivity (SYBR Green)



Supplier A

Copies

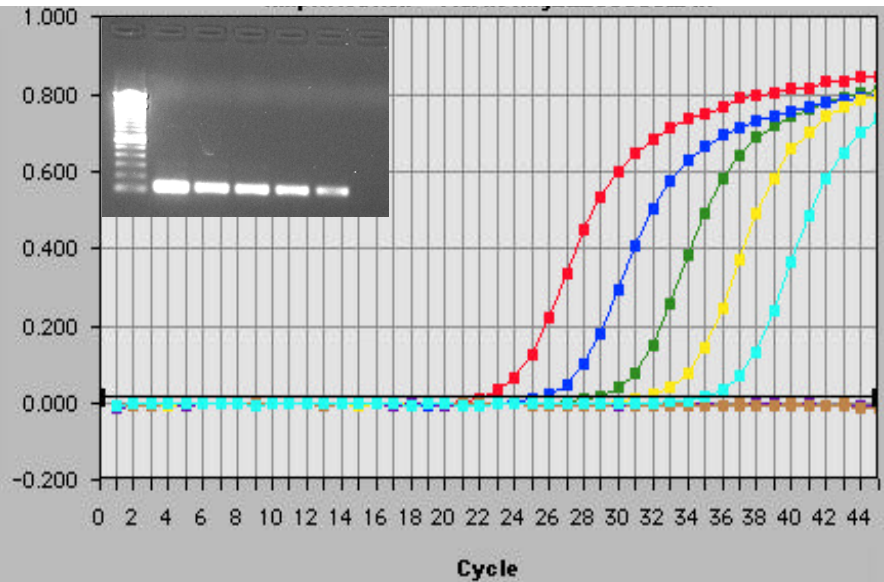
- $10^2$
- $10$
- $5$
- $0$



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# Improved Sensitivity in Probe-based Assays

## QIAGEN

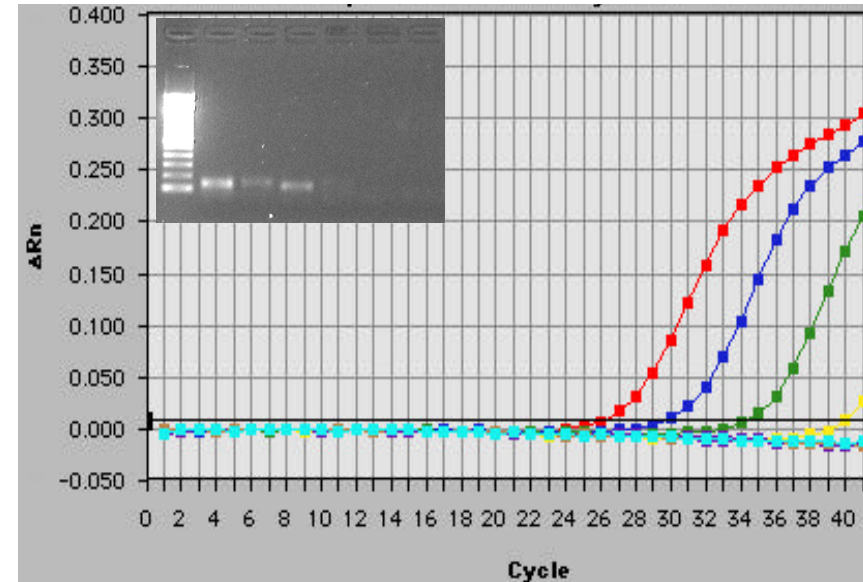


100 ng at  $C_T$  22.2

10 pg at  $C_T$  35.5

Efficiency: 98%

## Supplier A<sub>II</sub>



100 ng at  $C_T$  25.5

10 pg at  $C_T$  44.4

Efficiency: 89% (61%)

Gene Expression Assay Mm\_Bcl2

# QuantiTect PCR & RT-PCR Kits

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- Balanced combination of KCl and  $(\text{NH}_4)_2\text{SO}_4$   
→ Specific primer annealing during each cycle
- Stringent hot start with HotStarTaq DNA Polymerase  
→ High PCR specificity in initial PCR step
- Accurate & sensitive quantitation of transcripts
- Optimized for use with any real-time cycler
- Ready-to-use master mix format

# RT-PCR: The Reverse Transcription Step

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- Methods
- Efficiency, Sensitivity and Specificity
- Primers
- Template

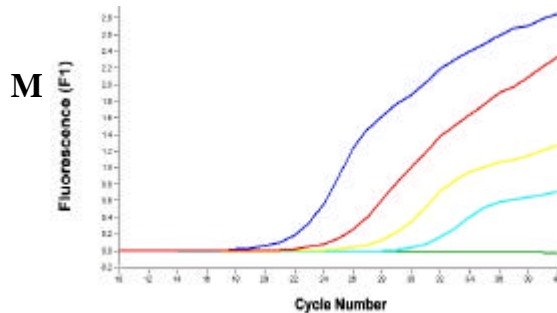
# One-Step and Two Step RT-PCR

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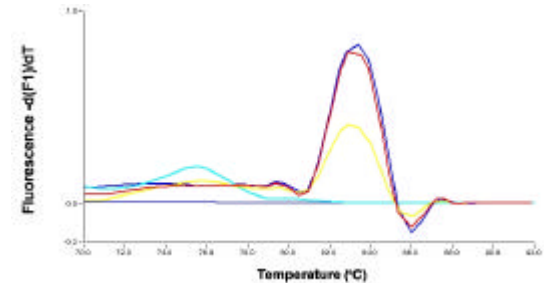
- One-Step RT-PCR
  - Single tube reaction
  - Direct link of both steps
  - RT starts from reverse PCR primer
  - Fast & reproducible procedure
  
- Two-Step RT-PCR
  - Two reaction setups
  - Temporally and physically separated
  - Various types of RT primers
  - 1 RT for multiple transcripts
  - Long-term storage of cDNA

# One Step RT-PCR: Problems and Solutions (I)

- Inhibition of PCR by RT enzyme



- ◆ Water
- ◆ 10 % mock RT
- ◆ 20 % mock RT
- ◆ 30 % mock RT
- ◆ No template control

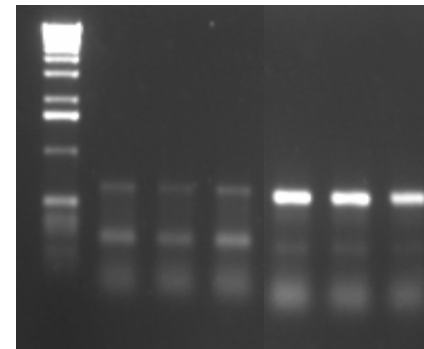


- Inhibition relief by

Optimized RT/PCR enzyme ratio

Additives (proprietary, patented technology)

- Additive    +Additive





# One Step RT-PCR: Problems and Solutions (II)

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- Efficiency and cDNA yield

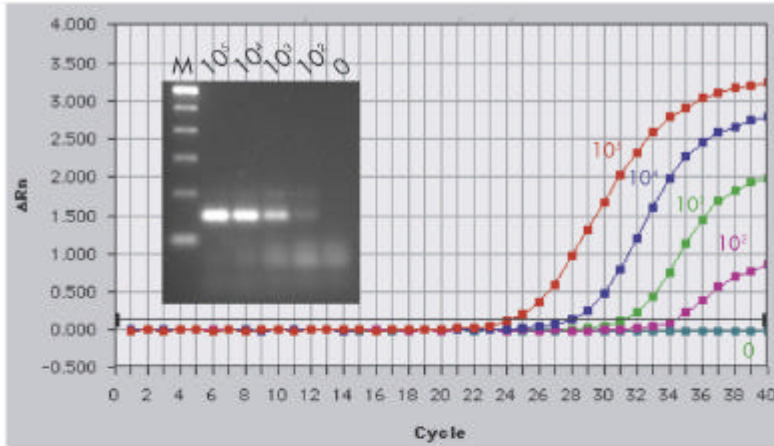
- High-affinity RT enzymes → No truncated cDNA
- Buffer additives → High RT temperature

- Specificity and Sensitivity

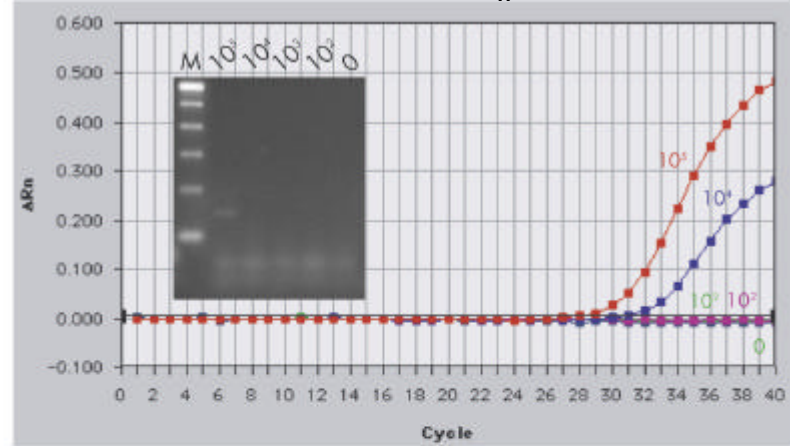
- HotStarTaq → No interference with RT step
- Balanced ion composition → High annealing specificity

# Reliable Quantitation in One-Step Real-time RT-PCR

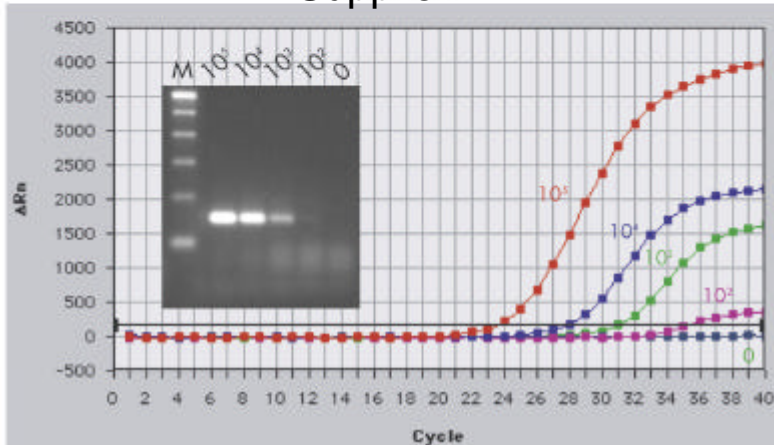
QIAGEN



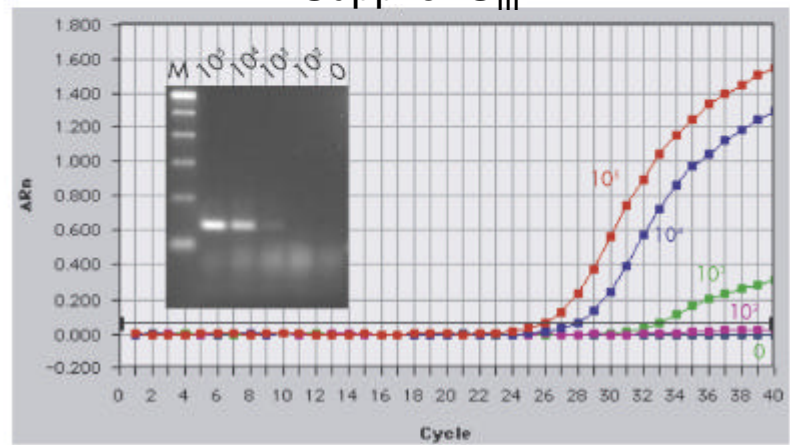
Supplier A<sub>II</sub>



Supplier I



Supplier S<sub>III</sub>

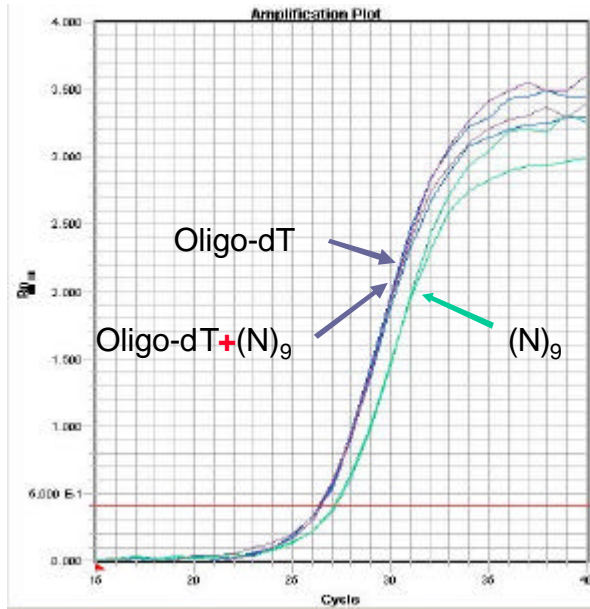


# Choice of RT Primer

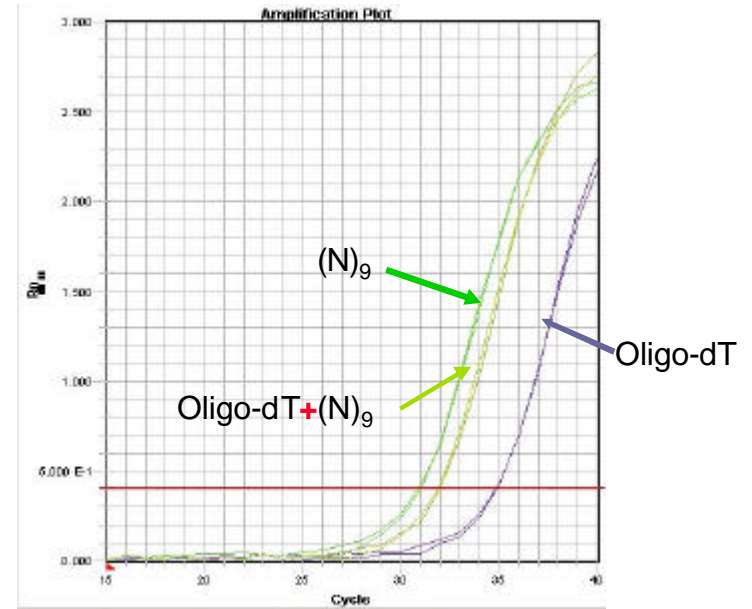
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- Selectivity Gene-specific (Oligo-dT)
- cDNA length Oligo-dT (Gene specific)
- Amplicon position Gene-specific (Random oligomers)
- Flexibility Oligo-dT + Random Oligomers

# Effect of RT Primer Choice



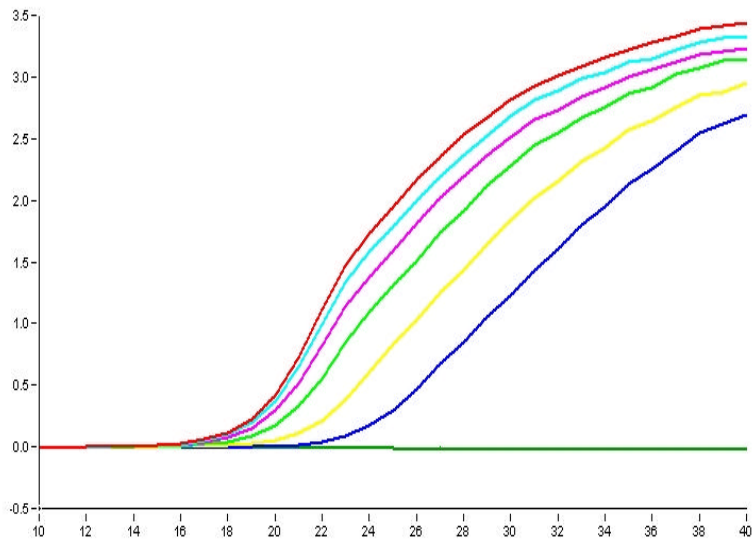
Amplicon – 3'-end: 2 kb



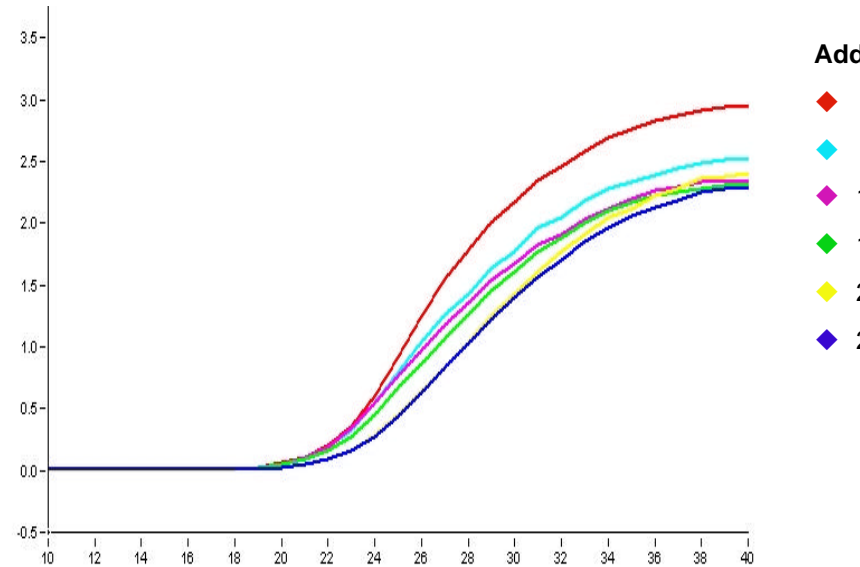
Amplicon – 3'-end: 6 kb

[Oligo-dT]	1 $\mu$ M
[(N) <sub>9</sub> ]	10 $\mu$ M
[Oligo-dT+(N) <sub>9</sub> ]	1 $\mu$ M/10 $\mu$ M

# Effect of RT Volume in Real-time PCR Reaction



Target A



Target B

# Summary: Reverse Transcription & Real-Time PCR

- High-affinity RT Enzymes and Buffer Additives  
→ Sensitive and linear one-step quantification
- Use of RT Primer Mixture  
→ High flexibility in amplicon choice
- Limited RT template volume  
→ Inhibition-free and reliable amplification

# QIAGEN R&D Group Modification/Amplification

